RELATIONSHIP OF BACTERIOPHAGES TO THE TOXIGENICITY OF *CLOSTRIDIUM BOTULINUM* AND CLOSELY RELATED ORGANISMS

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INTRODUCTION

The most important characteristic in the identification and differentiation of the pathogenic clostridia is the production of toxins. Based upon the production of antigenically specific neurotoxins, the species *Clostridium botulinum* is divided into types A through G. Even though the different toxin types represent a heterogenous group of strains, they have been placed into one species because of the similar pharmacological action of the toxins. When biochemical, physiological, and serological characteristics and deoxyribonucleic acid homologies are used to characterize the different *C. botulinum* strains, this species can be separated into four groups. Group I cultures are proteolytic and produce toxin types A, A_F, B, and F; group II cultures are nonproteolytic and produce toxin types B, E, and F; group III cultures are nonproteolytic and produce toxin types C_1, C_2, and D; and group IV cultures are weakly proteolytic and produce toxin type G.

The loss of the toxigenic characteristic has been observed in pure cultures of *C. botulinum* during culture in laboratory media. In addition, nontoxigenic clostridia resembling *C. botulinum* have been isolated frequently from aquatic and terrestrial environments. The occurrence of nontoxigenic

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cultures coupled with the observations that all types of *C. botulinum* carry bacteriophages (2,3,14,24) suggested that the production of toxins by *C. botulinum* might be mediated by bacteriophages or plasmids analogous to the production of toxin by *Corynebacterium diphtheriae* (1,10,11).

This report provides evidence for the involvement of specific bacteriophages in the toxigenicity of *C. botulinum* types C and D and closely related organisms.

BACTERIOPHAGES AND THE TOXIGENICITY OF *C. BOTULINUM* TYPES C AND D

C. botulinum types C and D produce at least three different toxins designated as C_1, C_2, and D (5,7,17). Type C strains produce predominantly C_1 toxin and minor amounts of C_2 and D toxins. In contrast, type D strains produce predominantly D toxin and minor amounts of C_1 and C_2 toxins. The minor toxins are not produced by all strains of types C and D.

The relationship of bacteriophages to the toxigenicity of *C. botulinum* was first observed in type C and D strains (4,6, 15,16). Nontoxigenic derivatives were isolated from toxigenic strains following acridine orange or ultraviolet irradiation treatments. When these nontoxigenic derivatives were infected with bacteriophages from the toxigenic parent culture, toxigenic isolates were again recovered.

The roles that different bacteriophage play in the toxigenicity and in the interrelationship of *C. botulinum* types C and D and closely related organisms are discussed in further detail in the following sections of this paper.

Toxigenicity of Type C Strains

A bacterial culture is generally immune to the infection by bacteriophages that it carries or to antigenically related bacteriophages that are produced by other cultures. In order to determine the relationship of bacteriophages to the toxigenicity of a bacterial strain, one must therefore isolate bacteriophage-sensitive derivatives, preferably from known toxigenic strains.

Strain 468C was the first culture used in our laboratory to study the involvement of bacteriophages in the toxigenicity of type C cultures. This strain was grown in trypticase, yeast-extract glucose (TYG) medium containing acridine orange (AO) or cultures in logarithmic phase of growth were treated with ultraviolet (UV) irradiation to cure them of their prophages. Surviving colonies that developed on TYG agar

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following anaerobic incubation were tested for sensitivity to the parent phages. After a 60-second treatment with ultraviolet light, 15 of 106 cultures tested were cured of prophages and concomitantly ceased to produce C_1 and D toxins. In comparison, 2 of 68 colonies tested from the acridine orange treatment were cured of their prophages and toxigenic characteristic.

The inability of these phage-sensitive cultures to produce C_1 and D toxins was confirmed during subsequent passages in laboratory medium and indicated that the loss of the toxigenic characteristic was permanent. Later, it was learned (5) that the production of C_2 toxin was not governed by bacteriophages and that phage-sensitive cultures continued to produce C_2 toxin. This toxin, however, was detectable only after activation with trypsin. For simplicity, the term "nontoxigenic" will be used hereafter in reference to cultures that failed to produce C_1 and D toxins.

To determine whether more than one bacteriophage was produced by 468C, each phage-sensitive "nontoxigenic" derivative was tested for its sensitivity to the lysates of other cured derivatives using the agar-layer procedure (4,6). Derivative AO28 was the only isolate that was sensitive to the lysates of other cured cultures. This culture had therefore been cured of two of its prophages. Colony-centered plaques (phage $1C^{tox+}$) and turbid plaques (phage $2C^{tox-}$) were produced on bacterial lawns of strain AO28 by phages isolated from cell-free lysates of the parent strain 468C. These phages were purified by five successive single-plaque isolations on strain AO28.

Table 1. Relation of Phages of Type C Strain 468C to Toxigenicity and Sensitivity of Strain AO28

Bacterial strain and phage	*Toxigenicity*[a]	*Sensitivity to phage*	
		1C	*2C*
AO28	-	+	+
AO28 (1C)	+	-	+
AO28 (2C)	-	+	-
AO28 (1C, 2C)	+	-	-

[a] *Production of predominant C_1 and minor D toxin*

The relationship of each of these phages to the toxigenicity of strain A028 was studied using procedures previously described (4). Table 1 summarizes the results of these experiments. When strain A028 was infected with phage 1C, it concomitantly produced dominant C_1 and minor D toxins and displayed immunity to infection by the homologous phage. Phage 2C, however, did not induce strain A028 to produce C_1 and D toxins, but the infected cultures, and all other cultures irrespective of phage involvement, did continue to produce C_2 toxin.

To determine whether the continued participation of phage 1C was necessary to maintain toxigenicity, strain A028 (1C) was cultured in TYG medium containing antiserum against phage 1C and plated on TYG agar. Isolates that were resistant to phage 1C continued to carry phage 1C and to produce C_1 and D toxins. On the other hand, isolates cured of phage 1C simultaneously ceased to produce C_1 and D toxins. These "nontoxigenic" isolates, however, resumed the production of C_1 and D toxins after they were reinfected with phage 1C. This curing and reinfection cycle was repeated with strain A028 and other "nontoxigenic" isolates from type C strain 468C and in every case the production of the C_1 and D toxins depended upon the continued participation of phage 1C. These results therefore emphasize the necessity of specific phages in the production of C_1 and D toxins by *C. botulinum* type C.

Strains of type C isolated from the different areas of the world were also examined to determine whether their phages also governed toxigenicity. In these experiments, spores from different strains were heated to 70°C for 15 minutes to inactivate free phage and plated on TYG agar. Vegetative cells were grown in TYG broth containing acridine orange or treated with ultraviolet irradiation and survivors plated on TYG agar. After anaerobic incubation, isolates were tested for phage-sensitivity and toxin production. All three methods yielded phage-sensitive derivatives which had simultaneously lost their ability to produce C_1 and D toxins. Strain 164 lost its phage and toxigenic characteristic during passage in EM medium (Table 2). Each of the "nontoxigenic" derivatives except the isolates from strain 6816 could be converted back to the toxigenic state (again produced C_1 and D toxins) when they were reinfected with specific TOX^+ bacteriophages from the toxigenic parent cultures. These converted cultures continued to produce TOX^+ phages and C_1 and D toxins during subculture in TYG or egg meat medium (EM). They also responded like type C strain 468C in being immune to the infection by the TOX^+ phages of the parent strain as long as they remained toxigenic and carried the corresponding TOX^+ phage.

With the exception of strain 162 or its phage-sensitive derivatives, all strains of type C produced the C_2 toxin

Table 2. Relation of bacteriophages to the toxigenicity of different strains of C. botulinum type C

Strain number	Method of obtaining cured cultures	Number of cultures			
		Tested	"Nontoxic"	Producing C_2 toxin	Converted to toxigenicity by phage
6816	Acridine orange	80	3	+	-
165	Spores	80	2	+	+
153	Spores	58	8	+	+
162	Acridine orange	64	2	-	+
162	Spores	40	1	-	+
3296	Spores	79	9	+	+
571	Spores	73	7	+	+
C_3	Acridine orange	92	9	+	+
C_8	Acridine orange	78	20	+	+
203	Acridine orange	89	5	+	+
2337	Acridine orange	102	40	+	+
6513	Acridine orange	160	86	+	+
SKM	Acridine orange	105	5	+	+
468C	Acridine orange	68	2	+	+
468C	Ultraviolet	106	15	+	+
460	Acridine orange	63	25	+	+
164	Passage in media	---	--	+	+

before and after they were cured of their TOX^+ prophages (Table 2). These results confirmed the earlier findings that the production of C_2 toxin was not governed by any of the bacteriophages used in these studies.

When the "nontoxigenic" isolates were tested for their sensitivity to the purified phages of the different type C cultures, five of the isolates were sensitive to numerous TOX^+ phages produced by toxigenic type C strains (Table 3). The remaining ten derivatives were sensitive only to the phages of the toxigenic parent culture. Each of the TOX^+ phages converted the "nontoxigenic" strains to the toxigenic state. Similar results have been reported with other type C strains (12,13,20-23). These results indicate that specific TOX^+ phages play a common role in the toxigenicity of different strains of type C.

Toxigenicity of Type D strains

The same procedures used to determine the involvement of bacteriophages in the toxigenicity of type C strains were also employed to study the toxigenicity of type D strains 1873 and South African. Strain 1873 produced the dominant D toxins and minor toxins C_1 and C_2. The South African strain, however, produced only the dominant D toxin.

Table 3. Host Range of Bacteriophages Isolated from C. botulinum Type C Strains

	Number of type C strains		
"Nontoxigenic" host	*Tested*	*Produced phage that infected "nontoxigenic" host*	*Converted "nontoxigenics" to toxigenic state*
AO50	*21*	*13*	*13*
AO28	*21*	*12*	*12*
HS46	*21*	*10*	*10*
HS31	*21*	*8*	*8*
HS34	*21*	*12*	*12*

When the South African strain of type D was studied, a greater number of "nontoxigenic" isolates were obtained from sporulated cultures than from vegetative cells cultures in TYG medium containing acridine orange. All of the "nontoxigenic" isolates from both sources were sensitive to phage $1D^{tox+}$ from the toxigenic parent culture. This phage converted each isolate to produce the dominant type D toxin. Toxigenic isolates continued to carry and to be immune to phage 1D.

Further studies were made with "nontoxigenic" isolate AO20. This isolate maintained its "nontoxigenic" state and sensitivity to phage 1D during numerous passages in EM medium over a 5-year period. It also maintained the toxigenic characteristic as long as it was infected with phage 1D. Strain AO20 (1D) was permitted to sporulate and the spores were washed, centrifuged, and plated on TYG agar. Following anaerobic incubation, colonies were again tested for their toxigenicity and phage-sensitivity. Of the 39 isolates selected, 19 were "nontoxigenic" and sensitive to phage 1D. After infection with phage 1D, each of the 19 isolates were converted to the toxigenic state and continued to produce type D toxin as long as they carried phage 1D. Occasionally, a toxigenic culture would become "nontoxigenic" during passage in EM medium. These "nontoxigenic" cultures were invariably sensitive to phage 1D and could be converted to the toxigenic state merely by phage infection.

These studies were also extended to type D strain 1873 to determine whether phages were involved in the toxigenicity of other type D strains that produce not only dominant D toxin but also minor C_1 and C_2 toxins. This toxigenic culture carried two phages designated as phage $2D^{tox+}$ and $3D^{tox-}$. Of 214 isolates examined from strain 1873 following acridine orange treatment, 23 were "nontoxigenic" and sensitive to phage $2D^{tox+}$. One of these isolates, AOA113, was also sensitive to phage $3D^{tox-}$. Phage 2D converted each of the "nontoxigenic" isolates to the toxigenic state and dominant D and minor C_1 toxins were again produced. Phage 3D, however, did not participate in the production of any of these toxins. All of the "nontoxigenic" and toxigenic isolates from strain 1873 continued to produce C_2 toxin which required trypsin activation to demonstrate toxicity.

Strain 1873 resembled the South African strain in that subcultures would occasionally lose their ability to produce D and C_1 toxins. These "nontoxigenic" cultures were always sensitive to phage 2D and could be converted to the toxigenic state by phage 2D.

Production of C_2 Toxin by Type C and D Cultures

Of the 21 different type C cultures isolated from six different countries, all except one produced C_2 toxin. This strain was isolated in England. The C_2 toxin from 15 of the strains cultured in EM medium required trypsin activation before toxin could be detected.

Type C cultures that had lost their toxigenic properties during transfer in laboratory media were received from other research laboratories labeled as "nontoxigenic" strains. Even though these strains did not produce C_1 and D toxins, 8 of the 15 strains did produce C_2 toxin which was detectable only after trypsin activation.

Recent studies indicate that the production of the C_2 toxin is correlated with the sporulation of type C cultures (18). The larger the sporulation, the higher the titer of C_2 toxin. When the spore populations were less than 10^4/ml of culture, C_2 toxin was not detectable in the culture supernatant fluids.

The optimum pH for trypsin activation of toxins from nonproteolytic strains of *C. botulinum* types B, E, and F is 6.0. When C_2 toxin was studied, the highest titers were obtained following trypsin activation at pH 6.5 (5).

Strain 1873 was the only type D culture that produced C_2 toxin. This toxin required trypsin treatment to demonstrate toxicity and was neutralized by antiserum prepared against the toxin of type C strain 468C. The C_2 toxins from type C and D strains therefore appear to be antigenically closely related (5).

INTERCONVERSION OF *C. BOTULINUM* TYPE C AND D STRAINS BY BACTERIOPHAGES

Strain 1873 was identified as *C. botulinum* type D because it produced the dominant D toxin. When strain 1873 was cured of phage $2D^{tox+}$, it could no longer be classified as type D because of its inability to produce D toxin. These "nontoxigenic" phage-sensitive derivatives, however, continued to produce C_2 toxin and became indistinguishable from "nontoxigenic" type C cultures.

The similarities in the characteristics of these cured derivatives of type C and D strains suggested that type C and D strains might arise from a common culture infected with different phages. To test this hypothesis, strain AOA113 was tested for its sensitivity to the phages of different type C strains. Phage $4C^{tox+}$ from type C strain 153 infected AOA113

and converted it to the toxigenic state in which C_1 toxin was dominant. When the cured derivatives of type C were tested for their sensitivity to phage $2D^{tox+}$ from 1873, only derivative HS15 from type C strain 153 was sensitive. Phage 2D converted HS15 to the toxigenic state and D toxin was dominant. As a result, derivatives HS15 and AOA113 became common hosts for both type D phage 2D and type C phage 4C. These cultures could therefore be converted to type D or to a type C merely by exchanging the TOX^+ phage (Table 5). Cultures infected with phage 4C were immune to infection by phage 2D and vice versa. Each culture irrespective of phage involvement produced C_2 toxin that required trypsin treatment to demonstrate toxicity.

Cultures AOA113(4C), AOA113(2D), HS15(4C), and HS15(2D) were permitted to sporulate and "nontoxigenic" derivatives were again isolated. These derivatives each became sensitive to phages 4C and 2D and when infected they again produced the dominant C_1 or D toxins, respectively.

These curing and reinfecting experiments were repeated three times and in each instance the production of toxin and the toxin type depended upon the continued presence of specific TOX^+ phages.

Interconversion of types C and D by bacteriophages was also observed in another group of strains that did not produce C_2 toxin. Strain HS37 (derived from type C strain 162) was not only sensitive to phage $3C^{tox+}$ of the parent strain but also to phage $1D^{tox+}$ from the South African strain of type D. Table 6 summarizes the results of the relationship of phage 1D and 3C to the type of toxin produced by strain

Table 5. Relation of Bacteriophages 2D and 4C to the Toxigenicity of Bacterial Strains AOA113 and HS15

"Nontoxigenic" cured cultures	*Phage*	*Number of cultures*		*Toxin neutralized by antiserum*
		Toxigenic and phage producers	*Tested*	
AOA113	*2D*	*20*	*20*	*Type D*
AOA113	*4C*	*37*	*37*	*Type C*
HS15	*2D*	*20*	*20*	*Type D*
HS15	*4C*	*20*	*20*	*Type C*

Table 6. Relation of Bacteriophages 1D and 3C to the Toxigenicity of Strain HS37

Infecting phage	Number of cultures		Toxin neutralized by antiserum
	Tested	Converted to toxigenic state	
1D	40	30	Type D
3C	40	40	Type C

HS37. Infection of HS37 with phage 3C resulted in the production of dominant C_1 toxin whereas infection with phage 1D resulted in the production of the dominant D toxin. Of 40 TYG cultures arising from plaque material from phage 1D, only 30 were toxigenic. The ten "nontoxigenic" isolates were retested and found to be phage-sensitive and capable of producing D toxin when they were infected with phage 1D.

Strain HS37(1D) produced only 10 MLD of D toxin per ml. When the culture supernatant fluid was treated with trypsin, the toxicity increased to 2000 MLD/ml. In contrast, the South African type D strain which also carried phage 1D produced 10,000 MLD/ml of type D toxin and the titer was increased only 10-fold by trypsin treatment. This difference in the toxicity suggests a difference in the enzymes produced by the two cultures.

Strain HS37 (1D) often lost its phage and reverted to the "nontoxigenic" state after three or four transfers in TYG or EM medium. The production of the D toxin could be restored by merely reinfecting the "nontoxigenic" isolates with phage 1D. The maintenance of phage 1D and toxigenicity by strain HS37 could be continued for longer periods of time when the EM medium contained 2% sodium chloride.

INTERSPECIES CONVERSION OF *CLOSTRIDIUM BOTULINUM* TYPE C TO *CLOSTRIDIUM NOVYI* TYPE A BY BACTERIOPHAGES

C. botulinum and *C. novyi* are pathogenic anaerobes that are characterized by their ability to produce powerful toxins.

The *C. botulinum* group produce neuroparalytic toxins that are responsible for botulism in man and animals. *C. novyi* also produce lethal toxins and are often found in gas gangrene infections of man and in other diseases of animals.

The species *C. novyi* includes a heterogenous group of organisms that is divided into types A, B, C, and D on the basis of different toxins produced. The production of lethal alpha toxin is the characteristic that unites types A and B. When types A and B strains were cured of their TOX^+ phages, they discontinued the production of the alpha toxin. As a result, "nontoxigenic" type A cultures no longer resembled the other *C. novyi* types, but instead became closely related to "nontoxigenic" *C. botulinum* type C and D strains (8,9). In comparison, when the *C. novyi* type B strains lost their TOX^+ phages and ceased to produce alpha toxin, they closely resembled *C. novyi* type D *(C. haemolyticum)* in that they continued to produce the same lethal beta toxin and other minor antigens. The main characteristic in the identification and differentiation of *C. botulinum* types C and D and *C. novyi* type A therefore is the toxins produced.

To determine the relationship of these two clostridial species, the phage-sensitive "nontoxigenic" derivatives of types C and D were tested for their sensitivity to the phages of 8 different strains of *C. novyi* type A. Strain HS37 (from type C strain 162) was found to be sensitive to the phages of *C. novyi* type A strain 5771. Cell-free lysates of strain 5771 contained two different phages. When phage $NA1^{tox+}$ infected strain HS37, the culture concomitantly produced the lethal alpha toxin of *C. novyi*. Phage $NA2^{tox-}$ also infected strain HS37, but showed no relationship to any of the toxins produced.

In earlier sections of this paper, strain HS37 was reported to be sensitive to type D phage 1D and type C phage 3C. The relationship of the phages NA1, 1D, and 3C to the toxigenicity of strain HS37 therefore was studied. When type C strain 162 was cured of phage 3C, it became "nontoxigenic" and a common host to phages NA1, 1D, and 3C (Table 7). Infection of strain HS37 with phage NA1 converted it to *C. novyi* type A and dominant alpha toxin was produced. If this culture was cured of phage NA1 and infected with phage 3C, then it was converted to *C. botulinum* type C, and the C_1 toxin was dominant. Phage-sensitive derivatives isolated from type C culture HS37 (3C) could then be infected with phage 1D and the culture was identified as type D because of the dominant D toxin. A phage-sensitive strain of clostridia therefore could be converted to *C. botulinum* type C or type D or to *C. novyi* type A by merely exchanging the bacteriophages. These

Table 7. Effect of Different Phage on Toxigenicity of Strain HS37

Phage	Number of cultures			Neutralized by antiserum of:
	Tested	Toxic	Produce phage	
$3C^{tox+}$	40	40	40	*C. botulinum type C*
$1D^{tox+}$	40	40	40	*C. botulinum type D*
$NA1^{tox+}$	40	40	40	*C. novyi type A*
$NA2^{tox-}$	40	0	40	----------

studies show that the toxigenicity of *C. botulinum* types C and D and *C. novyi* types A and B depends upon the continued participation of specific TOX^+ phages.

STABILITY OF PHAGE HOST RELATIONSHIP

The high frequency of isolating "nontoxigenic" phage-sensitive derivatives from toxigenic strains of *C. botulinum* types C and D and *C. novyi* types A and B following acridine orange and untraviolet irradiation treatments indicated that the phage-host relationship was unstable. Further evidence of this instability was obtained when isolates from toxigenic sporulated cultures were tested for their phage immunity and toxigenicity (Table 8). Even though the degree of instability varied markedly from strain to strain, all of the toxigenic strains yielded isolates that had lost their phages and immunity. These results suggested that a pseudolysogenic relationship existed between the phage and host.

In order to confirm these findings, toxigenic strains were transferred twice a day in TYG medium containing antiserum against the specific phages. Examples of the results are summarized in Table 9. A very high percentage of the isolates tested were phage-sensitive, and this percentage increased as the number of passages in phage antiserum increased. These results imply that the bacterial cells lose their phages during culture but are protected from reinfection by phage

Table 8. Loss of Phage and Toxigenicity through Spore State of C. botulinum Type C and C. novyi Type A

Strain	Number of colonies	
	Tested	"Nontoxigenic" and phage-sensitive
3296[a]	79	9
571	73	7
165	80	2
460	23	12
J C	97	9
162	40	4
SKM	30	6
C_3	40	4
C_8	20	20
203	89	5
X-200	40	39
2337	40	40
6513	86	16
468C	37	10
201	2	2
SA[b]	39	19
5771[c]	50	25

[a] *C. botulinum type C strain*
[b] *C. botulinum type D*
[c] *C. novyi type A*

antiserum. In the absence of antiserum, toxigenic cultures also lose their phages, but they can be reinfected by free phages which are present in an actively growing culture. These different results therefore support the fact that a pseudolysogenic relationship exists between phages and their host in *C. botulinum* types C and D and *C. novyi* type A and B strains.

Results from these studies demonstrate the important role that specific phages play in the production of *C. botulinum* C_1 and D toxins and the alpha toxin of *C. novyi* types A and B. Because of the pseudolysogenic relationship between the host and phage, these cultures occasionally lose their phages in nature and become "nontoxigenic." Depending on the presence

Table 9. Effect of Cultivation of Toxigenic Cultures in TOX+ phage antiserum on phage-sensitivity and toxigenicity

Strain	Number of transfers in antiserum	Number of cultures	
		Tested	Phage-sensitive and "nontoxigenic"
SKM[a]	3	40	6
	7	40	15
468C[a]	7	39	27
S.A.[b]	7	37	29
8024[c]	6	194	19

[a] *C. botulinum type C*
[b] *C. botulinum type D*
[c] *C. novyi type B*

of other phages, these "nontoxigenic" strains could be induced to produce *C. botulinum* toxins C_1 or D or the alpha toxin of *C. novyi*. Because of this role, these bacteriophages are very important to the identification of the pathogenic clostridia and also to the corresponding disease that they cause.

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